Characterization of *Pseudomonas aeruginosa* from chronic ear infections in dogs.

Introduction

P. aeruginosa is an opportunistic pathogen that causes a range of acute diseases in both humans and animals. In certain cases, such as in the airways of human cystic fibrosis (CF) patients and in the canine ear, *P. aeruginosa* may establish long, chronic infections. The infection process of the CF airways is associated with extensive genetic adaptation and evolution of the infecting *P. aeruginosa* bacteria over time. The accumulation of mutations results in strains with phenotypes of which many are not usually observed among environmental isolates. The occurrence of a range of genetic variants during chronic infections of the CF airways suggests within-host, parallel evolution of the infecting bacteria that secures their persistence and long-term survival. While chronic *P. aeruginosa* infections in CF patients have received considerable attention, very little is known of the mechanisms that allow *P. aeruginosa* to establish a chronic infection in other hosts and niches. However, we have recently shown that *P. aeruginosa* strains isolated from both from human and chronic canine ear infections exhibit phenotypes that are often associated with strains from CF patients. These traits include the appearance of different colony morphotypes (known as small colony variants - SCV) and the emergence of quorum sensing (QS) deficient mutants. In the case of CF lung infections, both SCV and QS mutants have been associated with a more severe course of the disease. We are working to identify the molecular nature of the SCV phenotype and the QS mutants. The finding of shared, system-independent phenotypes raises the possibility that the evolutionary pathways (or parts of them) that lead to a successful chronic colonizer may be parallel in different hosts and niches.

Description of the exercise

As a part of our research on *P. aeruginosa* infections, we have been following various CF patient groups and their infecting *P. aeruginosa* strains for several years (See supplementary papers). We have recently begun to investigate other chronically infected hosts – such as dogs. Ear infections in dogs are a common presenting problem in the clinical practice and may account for up to 20% of all consultations.

In this exercise, each team will analyze a pair of morphologically distinct *P. aeruginosa* strains isolated from an ear infection. All pairs of isolates that we will analyze in this exercise are from different hosts. We know very little of the isolates – except that the pairs are morphologically distinct, quorum sensing deficient and derived from the same infection site. On the basis of genotyping and relevant phenotype assays performed on these isolates each team will make new contributions to our understanding of the infection in these particularly fury patients.

Experimental keywords: genotyping using SNP arrays, small colony variants, antibiotic resistance profiling.

Relevant references:

Rau MH, Hansen SK, Johansen HK, Thomsen LE, Workman CT, Nielsen KF, Jelsbak L, Høiby N, Yang L, Molin S. Early adaptive developments of Pseudomonas aeruginosa after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. Environ Microbiol. 2010 Jun;12(6):1643-58. Epub 2010 Apr 7. PubMed PMID: 20406284.

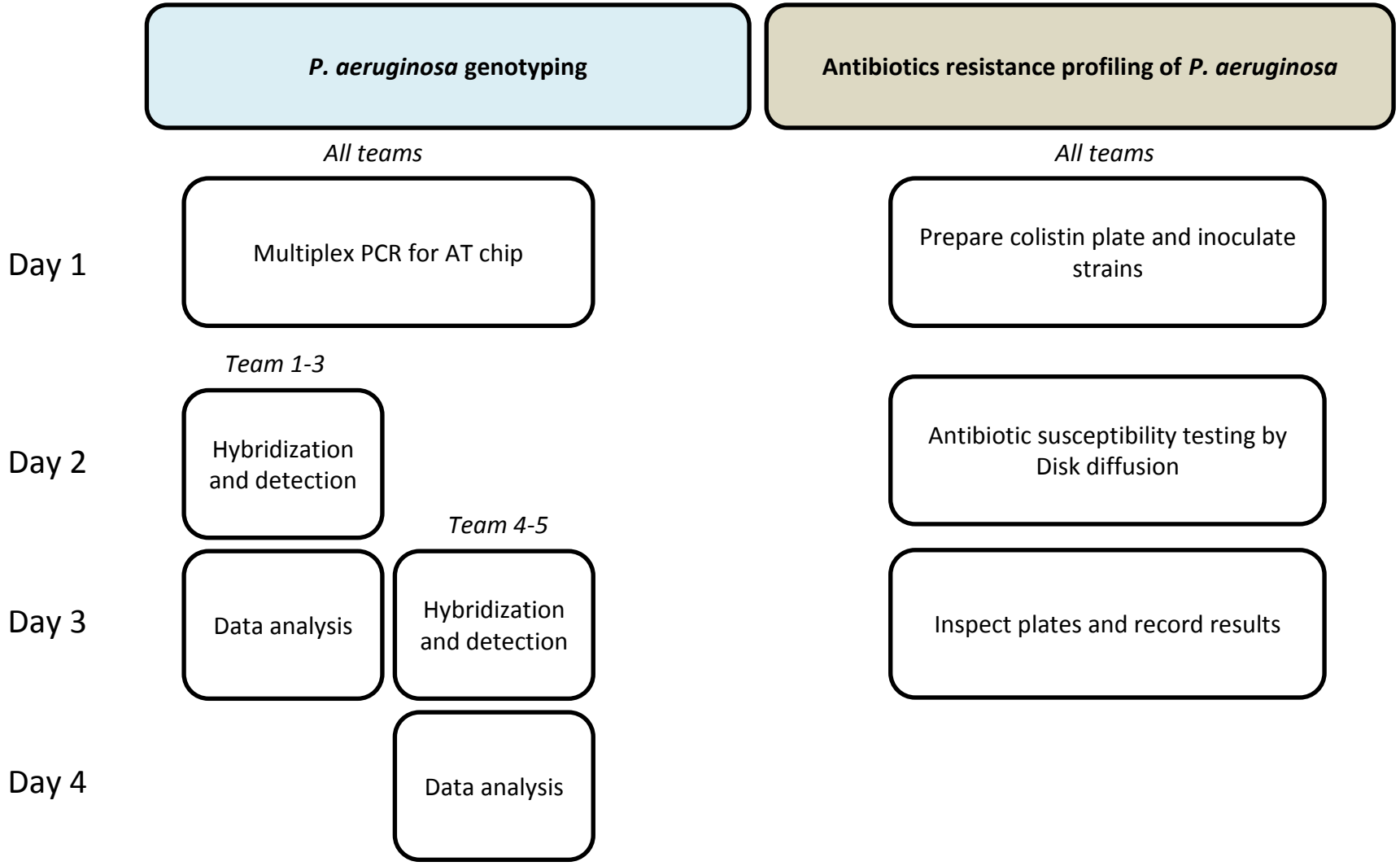
Jelsbak L, Johansen HK, Frost AL, Thøgersen R, Thomsen LE, Ciofu O, Yang L, Haagensen JA, Høiby N, Molin S. Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients. Infect Immun. 2007 May;75(5):2214-24. Epub 2007 Jan 29. PubMed PMID: 17261614; PubMed Central PMCID: PMC1865789.

Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, Palacios S, Manoil C, Kirisits MJ, Starner TD, Wozniak DJ, Harwood CS, Parsek MR. Pseudomonas aeruginosa rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J Bacteriol. 2009 Jun;191(11):3492-503. Epub 2009 Mar 27. PubMed PMID: 19329647; PubMed Central PMCID: PMC2681918.

Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, Köhler T, van Delden C, Weinel C, Slickers P, Tümmler B. Population structure of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2007 May 8;104(19):8101-6. Epub 2007 Apr 27. PubMed PMID: 17468398; PubMed Central PMCID: PMC1876578.

D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH, Brittnacher M, Hayden HS, Selgrade S, Klausen M, Goodlett DR, Burns JL, Ramsey BW, Miller SI. Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol. 2007 Apr;64(2):512-33. PubMed PMID: 17493132; PubMed Central PMCID: PMC2742308.

Overview of the experimental procedures



Experimental procedures

Day 1.

(A) *P. aeruginosa* genotyping

It is important that you have an understanding of the ArrayTube genotyping method before we start the experiments. Please consult appendix 3 and also the supplementary papers for this exercise – in particular the paper by Wiehlmann et al (2007).

Note: AT chip genotyping is performed on both isolates in independent reactions. The following protocol is described for 1 reaction

Preparation of bacteria

1. Swipe 2 loops of bacteria off the agar plate and resuspend in 100µl H_20 in an eppendorf tube.
2. Heat for 5min in a heating block at 99C
3. Cool on ice before using 8µl of the suspension for your PCR reaction (see next step)

Preparation of the PCR reaction:

1. Mix the following in a PCR tube:

10× Therminator Buffer	2.5 µl
8 mM dNTP (*)	2.5 µl
Primer mix	2.5 µl
DMSO	1.25 µl
Bacteria suspension	8 µl
Therminator polymerase	0.5 µl
Double-distilled H2O	7.75 µl
	= 25 µl

* 0.5 mM biotin-16-dUTP5 (Roche), 1.5 mM dTTP, 2.0 mM dATP, 2.0 mM dGTP, 2.0 mM dCTP.

2. Amplification protocol:

Initial denaturation 96°C 300 s
40 cycles 60°C 20 s
72°C 40 s
96°C 60 s
Cool down and store at 4°C

(B) Antibiotic susceptibility testing using Disk Diffusion

Preparation for tomorrow:

1. Inoculate a single colony of each of the 2 isolate in 10ml LB medium and incubate at 37C ON.
2. Inoculate a single colony of *Pseudomonas aeruginosa* PA01 (reference strain) in 10ml LB medium and incubate at 37C ON

3. We need to prepare colistin plates for "Antibiotic susceptibility testing using Disk Diffusion" tomorrow (Read appendix 1 to learn why):
   - Label an LB agar plate for each of your isolates
   - Place a colistin tab ("CO150") on each plate using forceps
   - After 2 hours at room temperature the tablet is removed (by knocking the plate against the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).
   - Outline the position of the tab with a pen on the backside of the agar plate.

Day 2.

(A) *P. aeruginosa* genotyping

****Important notes – read the following before you start the experiment***:*
- Do not centrifuge ArrayTubes (AT)
- Do not scratch or touch the array surface (e.g. during pipetting steps with the pipette tip)
- Avoid formation of air bubbles and remove them, if necessary, by mixing with a pipette
- Never let the AT run dry

Prepare Blocking solution
Mix 20mg Blocking powder with 1ml *hybridization buffer* in an eppendorf tube.

AT conditioning.
Prior to hybridization, the AT array needs to be conditioned:

1. Add 500 µl of *blocking solution* to the AT and incubate at room temperature for 5 min in a mixer at a mixing frequency of 550 rpm
2. Remove the *blocking solution* with a pipette (Do not touch the array surface!) and add 500 µl of *hybridization buffer*. Incubate at room temperature for 5 min a mixing frequency of 550 rpm.
3. Let the AT stand with the *hybridization buffer* until you are ready to add the hybridization mix (Step 6, below).

Hybridization.

4. Prepare Hybridization mix: Mix 20 µl of the PCR amplification product and 80 µl of *hybridization buffer* in a separate eppendorf tube (not inside AT).
5. Heat hybridization mix at 96°C for 5 min and chill on ice (this step is optional but may lead, in some cases, to stronger detection signals).
6. Remove the *hybridization buffer* from the AT (step 3, above) and transfer hybridization mix into AT and incubate at 60°C for 60 min in a thermomixer at a mixing frequency of 550 rpm.

Washing and blocking.

7. Remove *hybridization mix* from the AT and add 500 µl of *washing buffer I* at 30°C for 1 min in a thermomixer at a mixing frequency of 550 rpm.
8. Remove the buffer and add 500 µl *washing buffer II* at 20°C for 1 min in a thermomixer at a mixing frequency of 550 rpm.
9. Remove the buffer and add Wash AT array with 500 µl of *washing buffer III* at 20°C for 5 min in a thermomixer at a mixing frequency of 550 rpm.
10. Remove buffer and block unspecific binding by adding 100 µl of *blocking solution* and incubate in AT at 30°C for 15 min in a thermomixer at a mixing frequency of 550 rpm.

Staining and detection.

11. Remove *blocking solution* and add 100 µl of *conjugation solution* into the AT and incubate at 30°C for 15 min.
12. Remove the solution from the AT and add 500 µl of *washing buffer I* at 30°C for 1 min in a thermomixer at a mixing frequency of 550 rpm.
13. Remove the buffer and add 500 µl *washing buffer II* at 20°C for 1 min in a thermomixer at a mixing frequency of 550 rpm.
14. Remove the buffer and add Wash AT array with 500 µl of *washing buffer III* at 20°C for 5 min in a thermomixer at a mixing frequency of 550 rpm.
15. The AT array can be stored in this last washing buffer prior to detection for several hours).

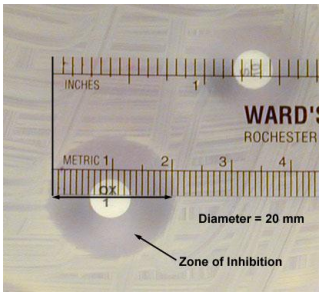
Image acquisition

16. Start computer, AT Reader and IconoClust-AT recording software.
17. Remove final washing solution and add 100 µl of 3,3',5,5',-Tetramethylbenzidine liquid substrate system for membranes (TMB) staining solution. Immediately insert the AT inside the AT reader and start image acquisition (note: the TMB-staining process is usually finished after 10 min).

Notes:
- Take more than just 1 image.
- The outside of the AT may be gently (!) cleaned using a piece of lens-paper with ethanol
- If there is "dirt" inside the AT, then remove the TMB solution (and the dirt) after you are done with the image acquisition, add 100µl of hybridization solution and acquire an extra image.

(B) Antibiotic susceptibility testing using Disk Diffusion

Susceptibility testing is of great importance to the hospital physician and the practitioner in the treatment of infectious diseases. Knowledge of susceptibility to different antibiotics of an infectious agent facilitates the choice of the most effective antibacterial compounds. In most clinical laboratories the *disk diffusion method* is used routinely for testing the susceptibility of bacterial pathogens. In this part of the exercise, you will test the susceptibility of your isolates to different clinically relevant antibiotics: Gentamycin, Enrofloxacin, and Polymyxins (*a.k.a.* colistin) (see the table below for the disks used in this exercise).



An example of antibiotic susceptibility testing using the Disk Diffusion method. You measure the diameter of zones of growth inhibition in millimeters. The zone seen in this example measures 20 mm in diameter.

Product	Code	Potency
Gentamycin	GEN40	40 µg
Gentamycin	GN250	250 µg
Enrofloxacin	ENROF	10 µg
Polymyxins (Colistin)	CO150	150 µg

1. Dry 1 LB plate per culture for 30min at 37°
2. Dilute each ON culture 10.000x in 0.9% NaCl and spread 100µl on an LB agar plate using a sterile “drigalski spatel”.
3. Place the 3 tabs on the agar plates using forceps
4. Incubate the plates at 35C ON.

Day 3:

(A) *P. aeruginosa* genotyping

Data analysis

For the data analysis you need the following items:

- The acquired images from your AT experiment
- The document “Short manual for analysis of AT chip data” (Appendix 5)
- The Excel sheet “AT chip skabelon” which can be found at the course web-site: *Supplementary Manuals/AT chip manuals and data analysis*.

At the course website you may also find an example of the data analysis

(B) Antibiotic susceptibility testing using Disk Diffusion

Inspect plates and record results in the data sheets on the following pages.

Group:

Results: Antibiotic susceptibility testing using Disk Diffusion

Name or number of Clinical isolate	GEN40		GN150		ENROF		CO150	
	Measured Zone diameter in mm	S, I, or R ? [(1)]	Measured Zone diameter in mm	S, I, or R ? [(1)]	Measured Zone diameter in mm	S, I, or R ? [(1)]	Measured Zone diameter in mm	S, I, or R ? [(1)]

Control:

PAO1								

[(1)] The isolate is **S**(sensitive), **I**(Intermediate) or **R**(resistant) toward the tested antibiotics. See "Zone diameter Interpretation Table" in appendix 2.

EUCAST-and CLSI potency
NEO-SENSITABS™
Performance of Susceptibility Testing

Pre-diffusion Method (2 + 18 or 2+22 hours) for Antimicrobials Diffusing Poorly on Agar

High molecular weight antimicrobials (vancomycin, teicoplanin, daptomycin, colistin) diffuse poorly on agar media, resulting in difficult to interpret results when using the disc diffusion method. Rosco Diagnostica has developed a 2 + 18 or 2+22 hour prediffusion technique, permitting an easier differentiation between susceptible and resistant strains when testing against these antimicrobials.

Procedure

One Neo-Sensitabs of the antimicrobial to be tested is placed on an uninoculated plate containing the susceptibility test medium (Mueller-Hinton plain or BHI Agar + 5 % blood).
After 2 hours at room temperature (≤25°C) the tablet (disc) is removed (by knocking the plate agianst the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).

The plate is now inoculated with the strain to be tested using a McFarland 0.5 inoculum.

Additional antibiotic discs (Neo-Sensitabs) may be added now using a dispenser (if MH agar is used) and thereafter the plate is incubated overnight at 35-37 °C.

The zones of inhibition are then measured. Zone breakpoints are tentative and for research use only.

Notice: In the laboratory, the prediffusion plate can be prepared the day before it is inoculated, in which case there is no loss of time and results are obtained within 24 hours.

NEO-SENSITABS™
Zone Diameter Interpretation Tables

	NEO-SENSITABS	POTENCY	CODE	Zone diameter in mm S	I	R	Break-points MIC µg/ml S	R
a) q)	Nalidixan (U)	130 µg	NALID	≥ 25	24-21	≤ 20	≤ 8	≥ 32
	Enterobacteriaceae			-	-	< 25	Reduced susceptibility to quinolones	
	Neomycin*	120 µg	NEOMY	≥ 25	24-21	≤ 20	≤ 6	≥ 25
	Netilmicin	40 µg	NETIL	≥ 20	19-17	≤ 16	≤ 12	≥ 32
a)	Nitrofurantoin (U)	260 µg	NITRO	≥ 23	22-20	≤ 19	≤ 32	≥ 128
a) q)	Norfloxacin (U)	10 µg	NORFX	≥ 16	15-14	≤ 13	≤ 4	≥ 16
i) *)	Novobiocin	**5 µg**	NOVO5	≥ 16	15-14	≤ 13	≤ 2	-
q)	Ofloxacin	10 µg	OFLOX	≥ 18	17-15	≤ 14	≤ 2	≥ 8
	Staphylococcus spp.			≥ 20	19-17	≤ 16	≤ 1	≥ 4
c)	Oxacillin	**1 µg**	OXA.1					
	S. aureus			≥ 13	12-11	≤ 10	≤ 2	≥ 4
	Coag. neg. staph.			≥ 18	-	≤ 17	≤ 0.25	≥ 0.5
a)	Oxolinic acid* (U)	10 µg	OXOLI	≥ 16	15-14	≤ 13	≤ 4	≥ 8
	Penicillin Low	5 µg	PEN.L	≥ 26	25-23	≤ 22		
b)	*Staphylococcus* spp.			≥ 26	-	< 26	≤ 0.1	Beta-Lactamase
e)	*Enterococcus* spp.			≥ 10	-	-	-	≥ 16
b)	Piperacillin	100 µg	PIPRA	≥ 23	22-20	≤ 19	≤ 16	≥ 128
h)	*Pseudomonas* spp.			≥ 18	-	< 18	≤ 64	≥ 128
d)	Piperacillin+Tazobactam	100+10µg	PI+TZ	≥ 23	22-20	≤ 19	≤ 16/4	≥ 128/4
h)	*Pseudomonas* spp.			≥ 18	-	< 18	≤ 64/4	≥ 128/4
s)	Polymyxins* (colistin)	150 µg	CO150	≥ 20	19-17	≤ 16	≤ 2	≥ 4
	Pristinamycin*	30 µg	PRIST	≥ 23	22-20	≤ 19	≤ 2	≥ 8

EUCAST-and CLSI potency NEO-SENSITABS™

Interpretation Zones and MIC Breakpoints according to CLSI

P. aeruginosa, Acinetobacter spp., B. cepacia, S maltophilia

PSEUDOMONAS AERUGINOSA

Zone diameter interpretative criteria and MIC breakpoints according to CLSI (formerly NCCLS) (1) when testing *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, *B. cepacia*, are listed in the tables below.

Table 3.2-1 Interpretation for *Pseudomonas aeruginosa*

Mueller-Hinton Agar. Inoculum McFarland 0.5. Incubation at 35 °C ± 2 degrees ambient air for 16-18 hours.

	NEO-SENSITABS	POTENCY	CODE	Zone diameter in mm S	I	R	Break-points MIC µg/ml S	R
a)	Amikacin	30 µg	AMI30	≥ 17	16-15	≤ 14	≤ 16	≥ 32
	Aztreonam	30 µg	AZT30	≥ 22	21-16	≤ 15	≤ 8	≥ 32
	Cefepime	30 µg	FEP30	≥ 18	17-15	≤ 14	≤ 8	≥ 32
d)	Cefepime+Clavulanate	FEP+C	30+10 µg	detection of ESBL				
	Ceftazidime	30 µg	CAZ30	≥ 18	17-15	≤ 14	≤ 8	≥ 32
d)	Ceftazidime+Clavulanate	CAZ+C	30+10µg	detection of ESBL				
	Ciprofloxacin	5 µg	CIPR5	≥ 21	20-16	≤ 15	≤ 1	≥ 4
c)	Colistin	10 µg	Co.10					
c)	2+18 hours' prediffusion method			≥ 15	14-11	≤ 10	≤ 2	≥ 8
	Doripenem	10 µg	DOR10	≥ 24	-	-	≤ 2	-
	Fosfomycin	200 µg	FO200	≥ 22	21-19	≤ 18	≤ 16	≥ 32
	Gatifloxacin	5 µg	GATIF	≥ 18	17-15	≤ 14	≤ 2	≥ 8
a)	Gentamicin	10 µg	GEN10	≥ 15	14-13	≤ 12	≤ 4	≥ 8
e) *)	Imipenem	10 µg	IMI10	≥ 22	21-18	≤ 17	≤ 4	≥ 16 per 30/4 2008
b)	Imipenem+EDTA	10+750µg	IM10E	detection of metallo-ß-lactamases				
	Levofloxacin	5 µg	LEVOF	≥ 17	16-14	≤ 13	≤ 2	≥ 8
e) *)	Meropenem	10 µg	MRP10	≥ 22	21-18	≤ 17	≤ 4	≥ 16
	Minocycline	30 µg	MIN30	≥ 19	18-15	≤ 14	≤ 4	≥ 16
	Netilmicin	30 µg	NET30	≥ 15	14-13	≤ 12	≤ 4	≥ 8
	Ofloxacin (U)	5 µg	OFL.5	≥ 16	15-13	≤ 12	≤ 2	≥ 8
	Piperacillin	100 µg	PIPRA	≥ 18	-	≤ 17	≤ 64	≥ 128
	Piperacillin+Tazobactam	100+10µg	PI+TZ	≥ 18	-	≤ 17	≤ 64/4	≤ 128/4
	Tetracyclines	30 µg	TET30	≥ 19	18-15	≤ 14	≤ 4	≥ 16
	Ticarcillin	75 µg	TIC75	≥ 15	-	≤ 14	≤ 64	≥ 128
	Ticarcillin+Clavulanate	75+10 µg	TIM85	≥ 15	-	≤ 14	≤ 64/2	≥ 128/2
a)	Tobramycin	10 µg	TOB10	≥ 15	14-13	≤ 12	≤ 4	≥ 8
	Dipicolinic acid	250 µg	D.P.A	Detection of metallo-β-beta lacta-mases				

- *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis requires incubation up to 24 hours.

*) *P. aeruginosa* low level resistant to meropenem (MIC 8 µg/ml) or Imipenem cannot be detected using the interpretation recommended by the CLSI. We recommend using Rosco's own interpretation.

Clinical isolates of *P. aeruginosa* heterogeneously resistant to carbapenems have been isolated (1). Subcolonies appearing within the zone of inhibition of Imipenem/Meropenem show higher MIC values. Automated systems and conventional agar dilution MICs using the standard 10^4 CFU per spot inoculum may miss carbapenem-resistant mutants.
Detection of *P. aeruginosa* resistant to Colistin in University Hospital in Barcelona (reference 3).

NEO-SENSITABS™ **Page 2 / 6 - Document: 10.1.0**

Zone Diameter Interpretation Tables **Enterobacteriaceae**

	NEO-SENSITABS	POTENCY	CODE	Zone diameter in mm S	Zone diameter in mm I	Zone diameter in mm R	Break-points MIC µg/ml S	Break-points MIC µg/ml R
	Clarithromycin	30 µg	CLARI	≥ 18	17-15	≤ 14	≤ 2	≥ 8
j)	Clindamycin	25 µg	CLIND	≥ 26	25-23	≤ 22	≤ 0.5	≥ 4
s)	Colistin	10 µg	CO.10					
	2+18 h prediffusion			≥ 15	14-11	≤ 10	≤ 2	≥ 8
v)	Daptomycin	30 µg	DAPCa					
	Staphylococcus spp. 2+18 h prediffusion			≥ 22	-	-	≤ 1	-
	Enterococcus faecalis (Vanco S) 2+18h pred.			≥ 12	-	-	≤ 4	-
u)	Doripenem	10 µg						
	Enterobacteriaceae			≥ 23	-	-	≤ 0.5	-
	P.aeruginosa			≥ 24	-	-	≤ 2	-
	A.baumannii			≥ 17	-	-	≤ 1	-
	Doxycycline	80 µg	DOXYC	≥ 20	19-17	≤ 16	≤ 4	≥ 16
q)	Enrofloxacin (Vet.)	10 µg	ENROF	≥ 23	22-17	≤ 16	≤ 0.5	≥ 4
c)	Ertapenem	10 µg	ERTAP	≥ 19	18-16	≤ 15	≤ 2	≥ 8
	-screen carbapenemases(Enterobacteriaceae)			-	-	< 22	Test for carba-penemase	
j)	Erythromycin	78 µg	ERYTR	≥ 26	25-19	≤ 18	≤ 0.5	≥ 8
a) l)	Fosfomycin (U)	70+40 µg	FOSFO	≥ 16	15-14	≤ 13	≤ 64	≥ 256
i)	Fucidin*	100 µg	FUCID	≥ 28	27-24	≤ 23	≤ 1	≥ 4
	Furazolidone*	50 µg	FURAZ	≥ 23	22-20	≤ 19	≤ 4	≥ 8
q)	Gatifloxacin	5 µg	GATIF	≥ 18	17-15	≤ 14	≤ 2	≥ 8
	Staphylococcus spp.			≥ 23	22-20	≤ 19	≤ 0.5	≥ 2
r)	Gentamicin	**40 µg**	GEN40	≥ 23	22-20	≤ 19	≤ 4	≥ 8
f)	Gentamicin	**250 µg**	GN250	-	-	< 14	-	> 500
	(Enterococci HLR all amino glycosides)							

ArrayTube (AT) chip genotyping overview

We apply molecular genotyping methods to monitor the *Pseudomonas aeruginosa* infection dynamics and epidemiology among CF patients. We primarily use the ArrayTube (AT) microarray from CLONDIAG to discriminate between clones isolated from infected patients. The AT technology is a DNA-chip based genotyping method in which labelled DNA is directly generated from a bacterial colony by multiplex PCR and then hybridized onto a microarray to yield a binary marker genotype (see Figure 1). We also use MLST and PFGE techniques for genotyping.

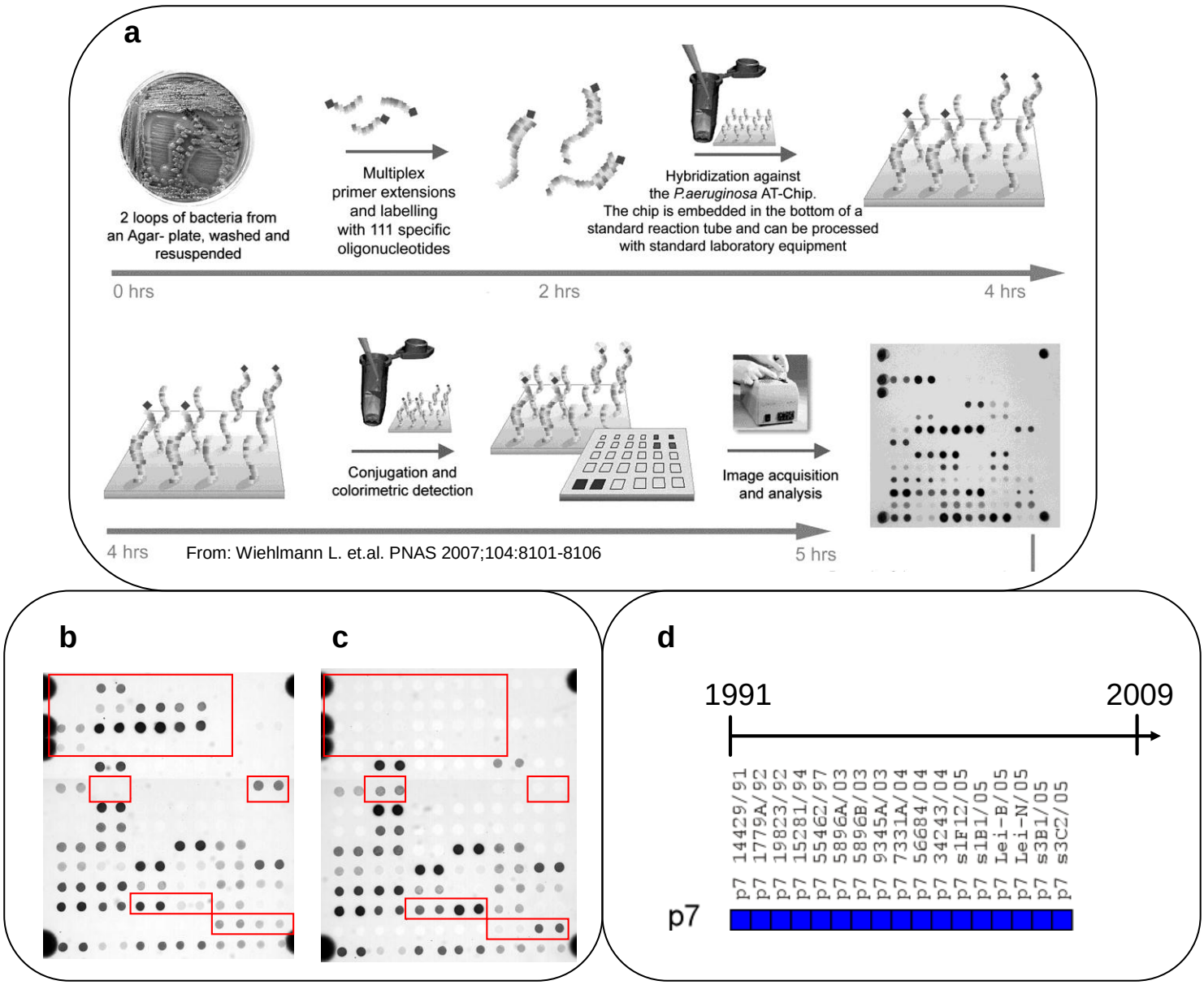


Figure 1. Molecular genotyping of *Pseudomonas aeruginosa*. **(a)** Outline of the experimental protocol for *P. aeruginosa* genotyping with the AT chip. **(b+c)** Examples of 2 different *P. aeruginosa* clones isolated from the same CF patient and genotyped using the AT chip. Red boxes indicate differences in the hybridization pattern. **(d)** *P. aeruginosa* infection dynamics in a single CF patient (patient "p7") with chronic *P. aeruginosa* lung infection since 1991. Genotyping of stored isolates showed that p7 had been infected with the same clone (indicated by blue squares in the figure) for the entire infection period. For details see Jelsbak et al (2007) Infect Immun. 75:2214-24.

Solutions used in Array Tube (AT) Genotyping of *Pseudomonas aeruginosa*.

20× SSC stock solution:	3 M NaCl, 0.3 M sodium citrate, pH 7.0
10× SSPE stock solution:	1.5 M NaCl, 0.1 M sodium phosphate, 0.01 M EDTA, pH 7.4
Mono-HRP-streptavidin concentrate:	1 mg/ml mono-HRP-streptavidin (Sigma-Aldrich, St. Louis, MO) in 50% glycerol
Bacteria washing buffer:	5 mM EDTA, pH 7.0
Hybridization buffer:	6× SSPE/0.1% (wt/vol) SDS
Conjugation solution:	1:100 dilution of mono-HRP-streptavidin concentrate in hybridization buffer
Blocking solution:	2% (wt/vol) blocking reagent (Roche Diagnostics, Basel, Switzerland) in hybridization buffer
Washing buffer I:	2× SSC/0.01% (vol/vol) Triton X-100
Washing buffer II:	2× SSC
Washing buffer III:	0.2× SSC

Short manual for analysis of AT chip data

1. Open the image-file of the AT chip result that you want to analyze: Open the image using e.g. 'picture-viewer'.

2. Make sure the image has the correct orientation: 3 control-spots in upper left corner (see figure 1). If the orientation is wrong it may be corrected by 'flipping' the image.

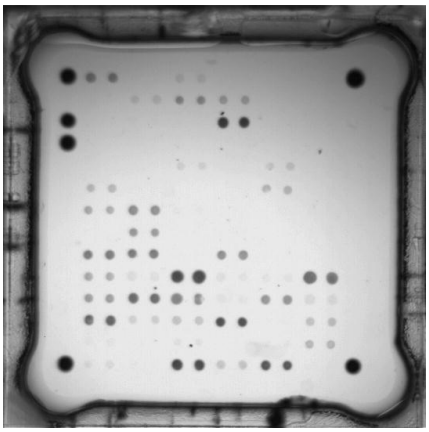


Figure 1. *An ArrayTube image. Note the dark control-spots in the corners of the chip.*

3. It is a good idea to make a print-out of the image for your files.

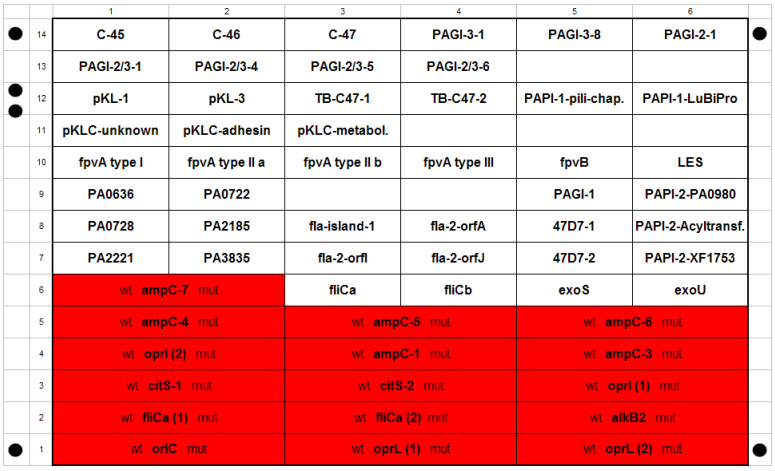
4. Print out the schematic outline of the AT chip (Figure 2 – See Figure 3 for a larger version).

5. Focus first on determining the SNP pattern. The SNP spots are found on the lower part of the AT chip (see Figure 3). With the help of the "SNP evaluation' figure (Figure 4) you can now determine whether the SNP are either 'wildtype' or 'mutant'. Record your results with a pen on the outline printout.

   NOTE: The 3 SNPs 'oprL', fliCa' and 'oprI' are measured twice on the AT chip.

   NOTE: Measurement of the 'fliCa' SNP is only meaningful when there is a signal in the variable gene 'fliCa' further up on the AT chip.

   NOTE: If you cannot determine whether it is a mutant or a wildtype signal (maybe there is no signal at all) then note that on the outline printout.



	1	2	3	4	5	6
14	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1
13	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6		
12	pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1-pili-chap.	PAPI-1-LuBiPro
11	pKLC-unknown	pKLC-adhesin	pKLC-metabol.			
10	fpvA type I	fpvA type II a	fpvA type II b	fpvA type III	fpvB	LES
9	PA0636	PA0722			PAGI-1	PAPI-2-PA0980
8	PA0728	PA2185	fla-island-1	fla-2-orfA	47D7-1	PAPI-2-Acyltransf.
7	PA2221	PA3835	fla-2-orfI	fla-2-orfJ	47D7-2	PAPI-2-XF1753
6	wt **ampC-7** mut		fliCa	fliCb	exoS	exoU
5	wt **ampC-4** mut		wt **ampC-5** mut		wt **ampC-6** mut	
4	wt **oprI (2)** mut		wt **ampC-1** mut		wt **ampC-3** mut	
3	wt **citS-1** mut		wt **citS-2** mut		wt **oprI (1)** mut	
2	wt **fliCa (1)** mut		wt **fliCa (2)** mut		wt **alkB2** mut	
1	wt **oriC** mut		wt **oprL (1)** mut		wt **oprL (2)** mut	

Figure 2. *A schematic outline of the AT chip. Note the control spots in the corners. The "SNP" part of the chip is highlighted in red*

6. Now you focus on the remaining spots (the variable genes). Use the outline printout and evaluate all spot. Simply record if you see a signal (This means that the gene is present). If you do no see a signal, then the gene is not there.

   NOTE: Make sure to note any problems with the present/absent determinations

7. Collect your data in an electronic form for further analysis

   For example, write your data into the Excel workbook called "*AT chip skabelon*". This self-explaining workbook is a good place to collect, store and analyze your data.

   Write the data into the "*Empty data sheet"* in this format:
   For the SNP data, type: '0' for wildtype, '1' for mutant, '-1' for undetermined.
   For the variable genes, type: '0' for absent and '1' for present.

   - The color of the cells in the excel sheet will change automatically.
   - An hexadecimal code will be calculated for each data-set.
   - Remember to write your notes/comments for each data-set
   - Use the "*cluster*" sheet to sort among different data sets.

Want help?!

1. Consult this AT chip analysis example: http://www.pnas.org/content/suppl/2007/04/18/0609213104.DC1/09213SuppAppendix.pdf
2. This paper describes the development and use of the ArrayTube genotyping method for *P. aeruginosa*: *Wiehlmann et al. (2007) Population structure of Pseudomonas aeruginosa. PNAS 104(19):8101-6.* http://www.pnas.org/content/104/19/8101.full.pdf

	1	2	3	4	5	6
14	**C-45**	**C-46**	**C-47**	**PAGI-3-1**	**PAGI-3-8**	**PAGI-2-1**
13	**PAGI-2/3-1**	**PAGI-2/3-4**	**PAGI-2/3-5**	**PAGI-2/3-6**		
12	**pKL-1**	**pKL-3**	**TB-C47-1**	**TB-C47-2**	**PAPI-1-pili-chap.**	**PAPI-1-LuBiPro**
11	**pKLC-unknown**	**pKLC-adhesin**	**pKLC-metabol.**			
10	**fpvA type I**	**fpvA type II a**	**fpvA type II b**	**fpvA type III**	**fpvB**	**LES**
9	**PA0636**	**PA0722**			**PAGI-1**	**PAPI-2-PA0980**
8	**PA0728**	**PA2185**	**fla-island-1**	**fla-2-orfA**	**47D7-1**	**PAPI-2-Acyltransf.**
7	**PA2221**	**PA3835**	**fla-2-orfI**	**fla-2-orfJ**	**47D7-2**	**PAPI-2-XF1753**
6	wt **ampC-7** mut		**fliCa**	**fliCb**	**exoS**	**exoU**
5	wt **ampC-4** mut		wt **ampC-5** mut		wt **ampC-6** mut	
4	wt **oprI (2)** mut		wt **ampC-1** mut		wt **ampC-3** mut	
3	wt **citS-1** mut		wt **citS-2** mut		wt **oprI (1)** mut	
2	wt **fliCa (1)** mut		wt **fliCa (2)** mut		wt **alkB2** mut	
1	wt **oriC** mut		wt **oprL (1)** mut		wt **oprL (2)** mut	

Figure 3. *A schematic outline of the AT chip. Note the control spots in the corners.*

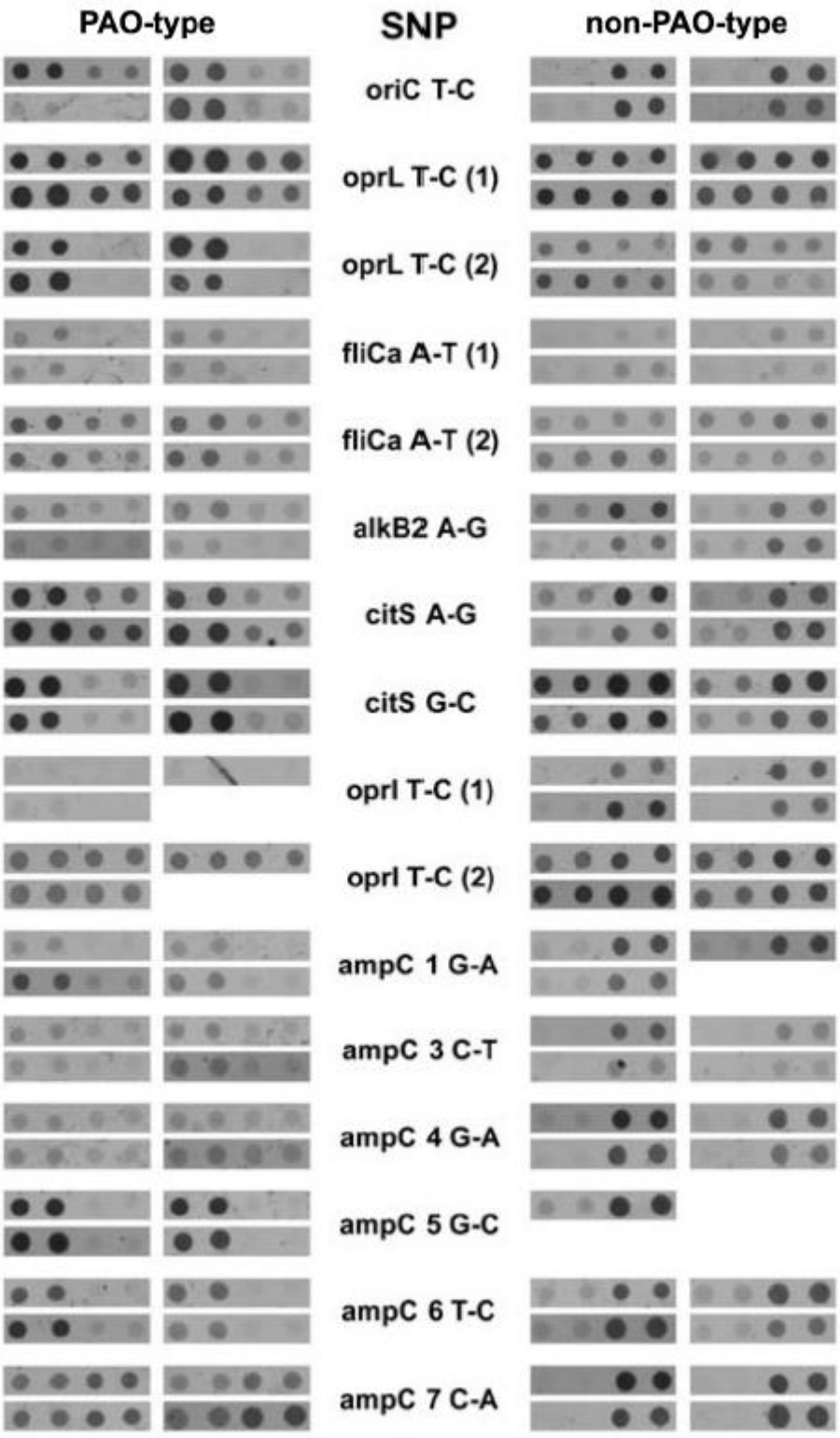


Figure 4. *Visual guide for the evaluation of the SNP pattern of the P. aeruginosa array tube array. For each SNP, PAO1-type and non-PAO1-type alleles are represented by several sets of primary hybridization data taken from separate strains and experiments*